Comparative in vitro evaluation of apheresis platelets stored with 100% plasma versus bicarbonated Ringer’s solution with less than 5% plasma

Shinji Oikawa, Dai Sasaki, Masaki Kikuchi, Yoshihiro Sawamura, and Takashi Itoh

BACKGROUND: The major strategy for reducing the frequency of adverse reactions to platelet (PLT) transfusions is PLT washing with PLT additive solutions (PASs). In Japan, a mixture of medical infusion solutions such as acetate Ringer’s solution, sodium bicarbonate, magnesium sulfate, and ACD-A is currently used as a PAS because none of the common types of PAS s are officially permitted for clinical use. Recently, a bicarbonated Ringer’s solution (BRS) was developed using bicarbonate as an alkaline agent. The aim of this study was to evaluate whether a BRS can effectively be utilized as a PAS for clinical use.

STUDY DESIGN AND METHODS: The washing and storage solution was prepared by adding 25 mL ACD-A to 500 mL of BRS (BRS-A), consisting of 95.2 mmol/L NaCl, 3.8 mmol/L KCl, 0.9 mmol/L MgCl₂, 1.4 mmol/L CaCl₂, 26.6 mmol/L NaHCO₃, 5.8 mmol/L glucose, 4.2 mmol/L trisodium citrate, and 1.8 mmol/L citric acid. The in vitro properties of apheresis PLTs suspended in BRS-A with low concentration of plasma (<5%) were compared with those suspended in 100% plasma during 7-day storage.

RESULTS: The in vitro properties of pH, hypotonic shock response, glucose consumption rate, lactate production rate, swirling, CD62P, and CD42b expression in PLTs suspended in BRS-A were comparable or superior to those suspended in 100% plasma during 7-day storage.

CONCLUSION: BRS-A, prepared by mixing the only two solutions permitted for clinical use in Japan, has a positive capability to maintain PLT function. These results indicate that PLT washing and storage with BRS-A is feasible.

Strategies to reduce the frequency of adverse reactions to platelet (PLT) transfusions, such as anaphylaxis and febrile nonhemolytic transfusion reactions, include 1) removal of the plasma supernatant from stored PLT concentrates (PCs) before transfusion and 2) prestorage reduction in white blood cell (WBC) levels to prevent WBC-derived cytokine accumulation during PLT storage. Several researchers have reported on the efficacy of plasma removal in this regard. Although volume-reduced PLTs are occasionally requested for patients with a history of severe allergic transfusion reactions, this technique may not remove a sufficient quantity of plasma to reduce the incidence of allergic reactions because volume reduction does not adequately remove plasma proteins. Therefore, PLT washing is currently recommended for patients with a history of severe allergic transfusion reactions.

PLT additive solutions (PASs) are used as plasma substitutes in several countries, and they confer a variety of roles, including a reduction in the amount of plasma required for PLT transfusion, recovery of additional plasma for plasma-derived products, avoidance of plasma-related transfusion reactions, and improvement in storage conditions. Current PAS varieties, such as PlasmaLyte A, T-Sol, Composol, InterSol, and SSP+, lack bicarbonate and glucose to avoid CO₂ egression and

ABBREVIATIONS: BRS = bicarbonated Ringer’s solution; BRS-A = BRS supplemented with ACD-A; HSR = hypotonic shock response; MPV = mean platelet volume; PAS(s) = platelet additive solution(s); PC(s) = platelet concentrate(s).

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Received for publication March 27, 2012; revision received May 21, 2012, and accepted May 21, 2012.


glucose caramelization, respectively. Consequently, it is imperative that a certain level of residual plasma be carried over to provide PLTs with bicarbonate and glucose.8 PLTs suspended in these PASs at low level of plasma (<20%) do not maintain their in vitro properties during a 5-day storage period.8 Recently, PAS-5, which is InterSol (PAS-3) reformulated by the addition of sodium bicarbonate, glucose, and other salts to better enable maintenance of in vitro qualities of PLTs with lower concentrations of plasma (approx. 5%), has been reported to maintain in vitro properties during a 7-day period.9 These facts support the hypothesis that bicarbonate and glucose are required to maintain the in vitro qualities of PLTs for longer periods in cases of PLT storage with low levels of plasma.

Currently in Japan, no PASs are officially permitted for clinical use in transfusions, despite the considerable advantages of having PLTs suspended in PASs, unlike plasma. Blood centers occasionally receive requests for washed PLT products to reduce the incidence of severe allergic or anaphylactic transfusion reactions in susceptible patients. The Japan Society of Transfusion Medicine and Cell Therapy has established guidelines for the processing of PCs, which include recommendations on several PASs prepared by the mixing of commercially available infusion solutions and a description of the standard manual technique for preparing washed PCs.10

The guidelines also recommend the use of a bicarbonate-buffered, glucose-containing PAS called M-sol, which has recently demonstrated a superior capability to preserve the in vitro qualities of PLTs in washed PCs with low concentrations of plasma (<5%) for at least a 7-day storage period.11,12 M-sol can be prepared manually by the mixing of commercially available infusion solutions. It has a superior capability of maintaining PLT functions. Accordingly, it has been under widespread use in Japan. However, M-sol preparation is laborious and includes complicated handling procedures, which is likely to affect the accuracy of its composition. Seeking commercially available sterile wash solutions that are less acidic, but provide PLTs with nutrients from the Japanese market, we selected bicarbonated Ringer’s solution (BRS). BRS was developed using bicarbonate as an alkaline agent instead of acetate.13 Commercially available BRS kits contain magnesium, glucose, and other electrolytes, packaged in a plastic bag with a gas-barrier film that maintains the stability of the solution by preventing carbon dioxide emission and pH elevation. BRS initiates a rapid acidosis correction, because sodium bicarbonate formulated as an alkaline agent does not require metabolic processing in vivo, providing physiologic bicarbonate by itself. In this study, we evaluated the in vitro properties of apheresis PLTs stored in BRS supplemented with ACD-A to determine whether it can be utilized as a PLT washing and storage solution for clinical studies.

### MATERIALS AND METHODS

#### BRS

BICANATE injection (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) consisting of 2.92 g NaCl, 0.15 g KCl, 0.11 g CaCl$_2$·2H$_2$O, 0.10 g MgCl$_2$·6H$_2$O, 1.175 g NaHCO$_3$, and 0.10 g Na$_3$-citrate·2H$_2$O in a 500-mL solution was used.

#### Anticoagulant supplementation of BRS

The washing solution for PLTs was prepared by adding 25 mL of ACD-A (Kawasumi Laboratories, Inc., Tokyo, Japan) to 500 mL of BRS (BICANATE injection, Otsuka Pharmaceutical Factory, Inc.; Table 1). After being gently mixed, BRS supplemented with ACD-A (BRS-A) was filter-sterilized using a sterile isolation bag with an integrated 0.22-µm filter (KBP-1000F, Kawasumi Laboratories, Inc.). The procedures above were performed just before PLT washing.

#### PLT washing

WBC-reduced apheresis PCs that failed to pass the alanine aminotransferase (ALT) test because of higher than permitted levels were used. The PLT content was calculated by multiplying the PLT concentration with the PC volume (mass/specific gravity, 1.03). The PCs were stored on a flatbed agitator (60 cycles/min) at 20 to 24°C and washed within 1 day after collection. After two ABO-identical PCs were mixed, the sample was divided into equal aliquots (BRS-A washing group and plasma group). For BRS washing group, PLT washing was performed by mixing the PCs with 250 mL of BRS-A and separated by centrifugation at 2560×g for 10 minutes at room temperature in a blood component centrifuge. Subsequently, the supernatant was pressed to the greatest extent possible with a manual hand press (separation stand, Kawasumi Laboratories, Inc.) into a previously connected bag. The bag containing the supernatant was disconnected, and BRS-A was added until the total volume reached 200 mL. The PLT pellets in the units were allowed to rest for 30 minutes and then resuspended on a flatbed agitator (60 cycles/min) at 20 to 24°C in polyolefin bags (KBP1000FPN, Kawasumi Laboratories, Inc.).

#### Table 1. Composition of BRS-A (mmol/L)

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>95.2</td>
</tr>
<tr>
<td>KCl</td>
<td>3.8</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>0.9</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>26.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.8</td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td>4.2</td>
</tr>
<tr>
<td>Citric acid</td>
<td>1.8</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Laboratory testing

Sampling was performed before and after PLT washing, as well as on Days 1, 3, 5, and 7 of storage. A 2-mL sample was obtained by sterile connection using an 80-mL sterile isolation bag (BB-T008FJ, Terumo Corp., Tokyo, Japan). The plasma protein levels of prewashed and postwashed PCs were measured by the bicinchoninic acid assay (BCA protein assay kit, Pierce, Rockford, IL). Before and after washing, the plasma protein content by volume was calculated to determine the percentage of protein removal, and the total PLT mass was calculated to determine the percentage of PLTs recovered.

The PLT count and mean PLT volume (MPV) were determined by using an automated blood cell counter (Sysmex KX-21, Sysmex Corp., Kobe, Japan). The pH, pO2, pCO2, and bicarbonate values were measured by using an automatic blood gas analyzer (ABL5, Radiometer, Copenhagen, Denmark) at 37°C. Commercial kits (glucose CII Test Wako, Wako Pure Chemical Industries Ltd., Osaka, Japan; l-lactic acid kit, Roche Diagnostics GmbH, Mannheim, Germany) were used to determine glucose and lactate concentrations (respectively) by colorimetric assay. For these assays, the PLT samples were pelleted upon centrifugation (5000 × g for 5 min), and the supernatant was withdrawn, aliquoted, and stored at −40°C.

Swirling was assessed by visual inspection and graded as 0 (no swirling), +, ++, or +++ (maximum swirling). Measurement of hypotonic shock response (HSR) required adjustment of the PLT concentration of the samples to 3 × 1011/L with AB plasma. The AB plasma, rather than the supernatant of the washed PCs, was used as a diluent to avoid the PAS readings contaminating the HSR measurement.14 The HSR at 5 minutes was determined by a standard method using a photometer (spectrophotometer DU640, Beckman Ltd., Tokyo, Japan).15

The surface expression of CD62P (P-selectin, GMP-140) and CD42b (glycoprotein Ibα) were detected by flow cytometry. Briefly, the PLT samples were fixed by dilution with 1% paraformaldehyde phosphate-buffered saline (PBS) at a ratio of 50:1000. The mixtures were stored at 4°C for more than 2 hours. After being washed twice with PBS, the samples were resuspended in PBS to obtain a PLT count of approximately 1 × 1010/L. For staining, 40 μL of the fixed samples was incubated with 10 μL of anti-CD62P-peridinin chlorophyll protein, 10 μL of anti-CD42b–phycoerythrin (PE), and 10 μL of anti-CD42b-fluorescein isothiocyanate (FITC) for 15 minutes in the dark at room temperature. Control specimens were processed as above, but incubated with two monoclonal immunoglobulin Gs (IgG): IgG1 PE–isotype control and a FITC-conjugated IgG1 isotype control. The reaction was terminated by adding 1 mL of PBS (stored at 4°C). All antibodies were purchased from BD Biosciences PharMingen (San Jose, CA). After being washed once, the samples were resuspended in 1 mL of PBS. These samples were then analyzed using a flow cytometer and its accompanying software (FACSCalibur and CellQuest Pro, respectively, Becton-Dickinson, San Jose, CA). Fluorescence data from 10,000 PLT events were collected in logarithmic mode. The PLT population was identified by CD61+ events. The population of activated PLTs expressing CD62P was defined by a minimum threshold set at 0.5% of fluorescence for the negative control sample.16 The results were expressed as specific CD61+/CD62P+ or CD61+/CD42b+ cells and as a percentage of all CD61+ cells.

Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using a computer program (MS Excel 2003, Microsoft Corp., Redmond, WA) with add-on software (Statcel 2, 2nd ed., OMS, Saitama, Japan). The statistical differences between the BRS-A washing group and the plasma group on each day were determined by a two-tailed paired t test. Differences were considered significant at p values of less than 0.05.

RESULTS

Residual plasma levels in BRS-A washing units were 2.1 ± 0.7%, and PLT recovery was 90 ± 1% (n = 5). The volumes in BRS-A and plasma were 206.7 ± 2.8 and 208.2 ± 3.5 mL, respectively (n = 5), on the washing day. A comparison of PLT storage variables for paired units suspended in 100% plasma (control) and washed with BRS-A (test) is presented in Tables 2 and 3. Although mean glucose levels of the test units were less than those of the control units during storage, the glucose consumption rate of the test units was comparable to that of the control units (0.95 ± 0.04 mmol/1012 PLTs/day vs. 0.67 ± 0.21 mmol/1012 PLTs/day; p = 0.059). The lactate production rate of the test units was comparable to that of the control units (1.85 ± 0.13 mmol/1012 PLTs/day vs. 1.64 ± 0.22 mmol/1012 PLTs/day; p = 0.271). The bicarbonate neutralization rate on Days 1 to 7 of the test units were comparable to that of the control units (1.60 ± 0.19 mmol/1012 PLTs/day vs. 1.33 ± 0.12 mmol/1012 PLTs/day; p = 0.0993).

DISCUSSION

We compared the in vitro properties (pH, pCO2, pO2, bicarbonate, MPV; glucose and lactate concentration; HSR; CD62P and CD42b expression; and swirling) in test and control units to evaluate the feasibility of BRS for PLTs washing and storage. The in vitro properties of PLTs in test units were comparable or superior to those of control units. These results indicate that BRS-A can be utilized as a PAS with a low concentration of plasma (less than 5%).

Volume 53, March 2013 TRANSFUSION 657
BRS-A has consisted of commercially available products, which are officially permitted for clinical use in Japan. Unlike previously reported PASs such as M-sol, PlasmaLyte A, T-sol, Composol, InterSol, SSP+, and PAS-5, BRS-A does not contain acetate, which has been the extent of shape change during storage. Furthermore, glycolysis, inhibit PLT activation, and maintain HSR and potassium and magnesium, which is well known to reduce pended with M-sol at low plasma levels. Heaton and coworkers reported the successful 7-day storage of PLTs by using bicarbonate, which suppresses pH decline by decomposing lactate into water and carbon dioxide.

Upon washing PLTs with BRS-A, PLT recovery was 90 ± 1%, which was comparable to or superior to the previous report showing high PLT recoveries. Veerapputhiran and coworkers demonstrated that PLT recovery is superior in saline-washed PLTs compared with PlasmaLyte A–washed PLTs, because saline washing results in less PLT clumping than PlasmaLyte A washing. In this study, almost no clumping was observed after washing procedures, which indicates that our cen-
trifugation conditions and the composition of BRS-A are suitable for PLT washing. In contrast, another study showed a higher PLT loss (approx. 41%-42.7%), which might have been caused by the suboptimal centrifugation conditions.25

Commercially available PASs, such as Composol, PlasmaLyte A, and SSP+, require some residual plasma in the washing preparations to provide PLTs with bicarbonate and glucose. However, Shimizu and coworkers26 reported that the in vitro properties of plasma-depleted PCs (%HSR, aggregation, supernatant β-thromboglobulin, and %discoid), which were washed by the Seto-sol, were maintained for 3 days at low plasma levels (approx. 0.9%). Recently, Azuma and coworkers26 demonstrated that PLTs suspended in M-sol at low plasma levels (3%-4%) significantly reduced adverse reactions and showed positive corrected count increments. BRS-A was developed with the aim of storing PLTs at low plasma levels (<5%). In this study, PLTs suspended in BRS-A at low plasma levels maintained in vitro properties during a 7-day storage period. The low residual plasma levels in BRS-A washing units are clinically acceptable for a patient with a history of transfusion reactions.

CD42b, an adhesion receptor on the surface of the PLT, binds to the von Willebrand factor (vWF), is expressed on resting PLTs and is reduced when PLTs are activated.27,28 CD62P translocates from the intracellular α-granule fraction to the PLT membrane surface in a manner dependent on the activation.29 Leytin and coworkers30 demonstrated that CD62P and CD42b expression is both related to PLT clearance in an animal model using rabbits. In this study, CD42b expression was stable in both the control and the test units during a 7-day storage period, suggesting that BRS washing does not affect the binding of PLTs to vWF. After a 3-day storage period, CD62P expression in the test units was significantly lower than that in the control units, which is most likely due to the presence of magnesium, potassium, and calcium and their role in inhibiting PLT activation in the test units.

The pH decline in the plasma (control) on Day 7 might be due to the exhaustion of bicarbonate in the sample. Hirayama and coworkers31 demonstrated that M-sol containing sufficient bicarbonate instead of sodium phosphate as a buffer led to a more stable pH. Similarly, bicarbonate in BRS-A is more likely to contribute to a stable pH during a 7-day storage period. The glucose in the BRS-A unit was almost exhausted on Day 5. Accordingly, lactate produced by anaerobic glycolysis was more likely to be suppressed after Day 5. The pH elevation on Days 5 and 7 might reflect the reduction of lactate production. On Day 7, low CD62P expression levels and high HSR in the test units indicate that the tricarboxylic acid cycle functions reciprocally for PLT viability, although glucose has been depleted.

The first limitation of our study is the lack of in vivo data. This study was performed using PCs rejected in the ALT test, but not yet expired, for in vitro analysis. According to a recent report that plasma-removed PCs suspended in M-sol in the presence of less than 20 mL plasma can be transfused safely,26 the use of washed PCs prepared by BRS-A could result in a lower frequency of adverse reactions. The second limitation is the relatively small number of experiments. However, the performance of the two-tailed paired t test proved the superior PLT storage capability of BRS-A.

In conclusion, our results indicate that BRS-A prepared by mixing only two solutions permitted for clinical use in Japan has a positive capability of maintaining PLT function. BRS-A is particularly useful in Japan, which has no officially permitted PASs to wash PLTs for clinical use.

ACKNOWLEDGMENTS

The authors thank Kenji Hirano, Satoshi Kosunago, Katsunao Tsukitake, and Takeshi Taguchi of Japanese Red Cross Miyagi Blood Center for their technical support.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest relevant to the manuscript submitted to TRANSFUSION.

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